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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07H 15/12 — A1

(11) International Publication Number: WO 91/17171

(43) International Publication Date: 14 November 1991 (14.11.91)

(21) International Application Number: PCT/US91/03139

(22) International Filing Date: 7 May 1991 (07.05.91)

(30) Priority data:
520,270 — 7 May 1990 (07.05.90) — US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: NUCLEOTIDE SEQUENCE ENCODING A 52 kDa Ro/SSA AUTOANTIGEN

(57) Abstract

Complementary DNA encoding a 52 kDa form of a protein present in the human Ro/SSA ribonucleoprotein complex has been cloned. A lambda gt11 cDNA library made from human thymus mRNA was screened with serum from an SLE patient and two immunoreactive clones were isolated. These clones reacted with other patient sera which had anti-52 kDa Ro/SSA antibodies and with affinity purified anti-52 kDa Ro/SSA antibodies. Moreover, affinity purified antibodies eluted from fusion proteins of the isolated clones reacted only with the 52 kDa protein of lymphocytes in the Western blot. Ro/SSA RNAs were also precipitated with these affinity purified antibodies, further confirming that the clones encode a 52 kDa Ro/SSA antigen. The sequence differs from the previously reported 60 kDa Ro/SSA gene. Both the cDNA and the protein expressed therefrom, or portions of either, are useful as diagnostic agents in the identification of patients having autoantibodies and in the identification and analysis of the structural and functional properties of the autoantigen and for application in immunotherapeutic regimens.

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NUCLEOTIDE SEQUENCE ENCODING A 52 KDA RO/SSA AUTOANTIGEN

Background of the Invention

The present invention is the nucleotide sequence encoding a 52 kDa Ro/SSA autoantigen found in some systemic lupus erythematosus and Sjogren's syndrome patients, the encoded protein and methods for use thereof in diagnostic and therapeutic applications.

Systemic lupus erythematosus (SLE) is similar to many other disorders in which autoantibodies are found and thought to be important in etiology and pathogenesis. SLE can be grouped with those diseases that commonly have autoantibodies present but for whom a central role of autoantibody in pathogenesis leading to clinical expression has yet to be fully established or accepted. Other such diseases include Sjogren's syndrome, rheumatoid arthritis, insulin-dependent diabetes mellitus, primary biliary cirrhosis, Wegener's granulomatosis, inflammatory bowel disease, polymyositis, dermatomyositis, scleroderma, and many others.

Typically, autoimmune diseases present with a wide array of symptoms and clinical signs. The production of circulating autoantibodies to ribonucleoprotein complexes (RNPs) is a unifying characteristic of some of the rheumatic autoimmune diseases. The most common antigens in SLE and closely related disorders include: Ro/SSA, La/SSB, nRNP and Sm. Initially, these antibodies were found using double immunodiffusion, but more recently sensitive solid phase assays have been developed to quantitate the autoantibodies. The Ro/SSA RNA-protein particle has been found to be a constituent of all human cells evaluated to date. Approximately half of Sjogren's syndrome and systemic lupus erythematosus (lupus) patients have anti-Ro/SSA precipitins. Approximately 75% of patients with subacute cutaneous lupus erythematosus or complement component C2 deficiency with SLE have anti-Ro/SSA precipitins, and virtually all patients with

C2 or C4 deficiency have elevated levels of anti-Ro/SSA when measured by ELISA. Over 80% of mothers of newborns with neonatal lupus dermatitis or complete congenital heart block have these autoantibodies. As many as 5% of patients with rheumatoid arthritis, polymyositis, and progressive systemic sclerosis have anti-Ro/SSA, as reported by R.M. Bernstein, et al., Mol. Biol. Med. 2:105-120 (1984); and J.B. Harley and K.K. Gaither, Autoantibodies. In Rheumatic Disease Clinics of North American: Systemic Lupus Erythematosus 14:1, 43-56 (1988).

It has also been shown that some normal individuals have low levels of anti-Ro/SSA, that some normal family members of SLE patients have anti-Ro/SSA, and that 1% of normal pregnant women, and 0.1% of a cohort of hospitalized patients have precipitating levels of this autoantibody (K.K. Gaither, et al., J. Clin. Invest. 79:841-846 (1987); T.J.A. Lehman, et al., J. Rheumatol. 11:644-647 (1987); M. Calmes and B.A. Bartholomew, J. Clin. Pathol. 38:73-75 (1985); P.J. Maddison, et al., J. Rheumatol. 5:407-411 (1978)). Even if the anti-Ro/SSA autoantibody is not pathogenic, the concentrations of anti-Ro/SSA autoantibody achieved by patients can be extraordinary, and is commonly higher than 1 mg/ml of specific anti-Ro/SSA immunoglobulin (K.K. Gaither and J.B. Harley, Prot. Biol. Fluids Proc. Colloq. 33:413-416 (1985); J.B. Harley, et al., Arthritis Rhuem. 29:196-206 (1986)). The immune system derangement leading to this specific overproduction of anti-Ro/SSA is not apparent but is likely to reflect a fundamental mechanism related to the immunopathogenesis of the related diseases.

Ro/SSA has been referred to by several other names, including "SSA/Ro", "SS-A/Ro", "SS-A", "Ro", and "Ro(SSA)". Historically, the biochemical characterization of the Ro/SSA complex has centered

around a 60 kDa protein associated with one of four hY RNAs, ranging from 80 to 112 bases, although the antigenic reactivity of the complex appears to be independent of the RNA. The Ro/SSA family of proteins has now been shown to have several molecular forms which are operationally defined by the molecular weight of the antigen identified. As reviewed by Ben-Chetrit, et al., in J. Exp. Med. 167, 1560-1571 (1988), the protein components of Ro/SSA have been described as polypeptides having molecular masses ranging from 50 to 150 kiloDaltons (kD). A major form has an apparent molecular weight of 60 kDa. Recently, two additional proteins bound by anti-Ro/SSA sera have been identified by M.D. Rader, et al., J.Clin. Invest. 83:1556-1562 (1989), with molecular weights of 52 kDa and 54 kDa. Ben-Chetrit, et al., (1988) also report a 52 kDa protein. Chan, et al., reported at the Molecular and Cell Biology of Autoantibodies and Autoimmunity, First International Workshop, July 27-29 (1989), that they had cloned a gene encoding a 46 kDa protein reactive with antisera against a 52 kDa Ro/SSA protein that was distinct, based on sequence comparison, from the cDNA of 60 kDa Ro/SSA. Other groups have confidentially reported that they have isolated cDNA encoding a different 60 kDa protein, having a molecular weight predicted by sequence analysis of 48 kDa.

It is impossible to determine at this time how many different autoantigens are produced which form complexes with RNA and that are characteristic, or involved in the pathogenesis, of autoimmune disorders in humans, such as SLE and Sjogren's syndrome. The proteins may vary not only from patient to patient, but in cellular origin. For example, in nucleated cells, 60, 52 and perhaps 45 kDa forms of the Ro/SSA protein have been found using Western blot

analysis. Certain lupus patient sera contain antibodies which recognize only the 60 kDa form, others only the 52 kDa form, and others have antibodies which bind to both the 60 and 52 kDa forms. In red blood cells, 60 and 54 kDa proteins have been identified in Ro/SSA particles. It appears that these proteins can only be identified with any certainty by comparison of nucleotide and amino acid sequence comparison.

It is therefore an object of the present invention to provide cDNA encoding an autoantigen characterized by a molecular weight of approximately 52 kDa to which serum antibodies are produced by certain patients having autoimmune disorders.

It is a further object of the present invention to provide the cDNA and the protein encoded by the cDNA and methods for use thereof for diagnostic and therapeutic purposes.

Summary of the Invention

Complementary DNA encoding a 52 kDa form of a protein present in the human Ro/SSA ribonucleoprotein complex has been cloned. A lambda gt11 cDNA library made from human thymocyte mRNA was screened with serum from a SLE patient and two immunoreactive clones were isolated. These clones reacted with other patient sera which had anti-52 kDa Ro/SSA antibodies and with affinity purified anti-52 kDa Ro/SSA antibodies. Moreover, affinity purified antibodies eluted from fusion proteins of the isolated clones reacted only with the 52 kDa protein of lymphocytes in the Western blot. Ro/SSA RNAs were also precipitated with these affinity purified antibodies, further confirming that the clones encode a 52 kDa Ro/SSA antigen. The sequence differs from the previously reported 60 kDa Ro/SSA gene.

Both the cDNA and the protein expressed therefrom, or portions of either, are useful as diagnostic and therapeutic agents in the identification and treatment of patients having autoantibodies and in the identification and analysis of the structural and functional properties of autoantigens reactive with antibodies to the 52 kDa protein. The cDNA is also useful in the isolation of nucleic acids encoding related proteins. The related proteins can be expressed from these sequences and are also useful as diagnostic and therapeutic agents.

Brief Description of the Drawing

Figure 1 shows the cDNA sequence for the Ro/SSA 52 kDa protein.

Figure 2 shows the predicted amino acid sequence of this gene. The predicted size of the polypeptide encoded by this sequence is 54,108 daltons.

Detailed Description of the Invention

cDNA encoding a protein having a molecular weight of 52 kDa based on SDS-PAGE and immunological identification in Western blots and a predicted molecular weight of 54,108 daltons has been cloned. The cDNA sequence is shown in Figure 1 and the encoded amino acid sequence is shown in Figure 2. The sequence is distinct from any published sequence, including that for two different sequences which may encode the 60 kDa Ro/SSA protein, providing further support for the heterogeneity of this group of proteins. There is similarity between the amino-terminal portions of this predicted protein and the amino-termini of both the mouse rpt-1 protein, a T cell regulatory protein, and the predicted human ret/rfp protein. The level of identity between the

amino terminal portion of this cloned protein and the latter two sequences are 48 and 43%, respectively. This area in the three proteins include zinc finger motifs. Further similarity is found between the carboxy-terminal ends of the predicted protein for this 52 kDa Ro/SSA molecule and the human rfp protein (51% identity), suggesting that the gene encoding this Ro/SSA protein may be a member of a larger gene family. It is also possible that polymorphisms of this gene will be identified, i.e. genetic differences in the nucleotide sequence of this gene may exist in humans, perhaps between patients and healthy controls, or in a subset of patients. These polymorphic genes and their protein products (and any of their modified forms) can be identified and isolated using either the disclosed cDNA sequence or protein expressed from the sequence, using techniques known to those skilled in the art, such as those described by Maniatis, et al., Molecular Cloning. A Laboratory Manual (Cold Spring Harbor Laboratory, NY 1982). For example, the related sequences can be identified and isolated by hybridization under standard conditions to a probe having a sequence shown in Figure 1 (65°C in the absence of foramide, with unbound probe removed by washing with decreasing concentrations of from 1 to 2 x SSPE to 0.5 x SSPE).

Referring to Figure 1, the first methionine residue is encoded by an ATG codon beginning at position 39. A stop codon (TGA) is found at position 1463. A putative poly-adenylation recognition site (AATAAA) is located at position 1827. This normally occurs approximately 20 bases upstream of poly-A sequences on eukaryotic mRNAs. Thus a 414 base 3' untranslated region occurs on this molecule. The enzyme EcoRI, which has a recognition sequence

GAATTC occurring at the beginning and end of this sequence, cleaves this cDNA just short of the poly-A sequence.

As shown in Figure 2, this predicted molecular weight of the encoded protein is slightly larger than the experimentally determined size of the 52 kilodalton protein. Post-translational processing or unusual secondary structure of this gene product is hypothesized to result in the 52 kDa band detected on Western blots.

Protein can be expressed from the cDNA using standard techniques for expression *in vitro* in cell free translation systems, in bacteria, yeast, and animal cells, including insect, amphibian, avian, and mammalian cells, as well as genetically engineered animals. The techniques are known to those skilled in the art. Reagents, including expression vectors and cell lines, for use in these methods are commercially available.

It is understood that specific cDNA sequences can be modified by those skilled in the art, for example, by labelling, fusion with regulatory sequences, insertion into expression vectors, site-directed mutagenesis and substitution or deletion of nucleotides encoding specific amino acids, without departing from the scope of the nucleotide and amino acid sequences of the present invention, and the methods for their use.

There are several embodiments of diagnostic reagents using the cDNA and protein expressed therefrom, in whole or in part, that can be used for diagnosis of autoimmune disorders or presence of autoantibodies. Antibodies to the protein obtained either from patients, immunized animals, or from antigen-specific monoclonal cell lines can be used to detect the respective antigen in cell extracts or serum and body fluids. These antibody assays include assays such as sandwich

ELISA assays, Western immunoblot, radioimmunoassays, and immunodiffusion assays. Techniques for preparing these reagents and methods for use thereof are known to those skilled in the art.

Expressed protein can also be used to immunize animals to generate polyclonal antisera and/or monoclonal antibodies. These, as well as patient autoantibodies, can be used to analyze structure and function of this protein. Protease cleaved fragments of the expressed proteins, or synthetically produced oligopeptides generated from the predicted sequence can be isolated and used to detect autoantibodies, as well as determine the epitope structure of the autoantigens. The expressed protein, or modifications thereof as described above, can be used to purify antibodies from different patients in order to study the heterogeneity of this autoimmune response, and if such heterogeneity is found, to appreciate the relationship between such heterogeneity and disease differences in these patients.

Nucleotide or amino acid probes can be prepared based on the sequence in Figure 1. These are labelled using dyes, or enzymatic, fluorescent, chemiluminescent, or radioactive labels which are commercially available. These probes can be used to detect the expression of this gene or related sequences in cells, tissue samples, or in *in vitro* reagents, as well as to screen sera or tissue samples from humans suspected of having autoantibody. For example, the appropriate nucleic acid sequences (or their complementary nucleic acid) could be used for *in situ* hybridization as a method to detect expression of genes encoding the antigen for the autoantibodies in specific tissues or peripheral blood cells. Levels of gene expression can be quantitated in patients and compared to healthy controls, or can be compared between different tissues. Differential tissue expression, coupled with data on

tissue pathology and detection of anti-52 kDa antibodies in these tissues, may be used to analyze the relation between antibody binding *in vivo* and disease pathogenesis. Nucleic acid primers could also be prepared which, with reverse transcriptase or DNA polymerase and the polymerase chain reaction, could be used to expand prospective antigenic sequences. Polymorphisms of this gene may be detected following restriction enzyme digestion of cellular DNA with subsequent electrophoresis and transfer of DNA to a membrane. Products of alleles to the sequence reported in Figure 1 may bind to patient autoantibodies with different affinities or may cross-react with epitopes present on other particles. The cDNA probe can also be used to clone the 52 kDa Ro/SSA gene from chromosomal DNA from which questions regarding differential splicing of exons and gene regulation can be addressed. Alterations of the DNA by site-directed mutagenesis may be used to determine functional regions including those which bind to hY RNAs.

Therapeutic applications of the cDNA and proteins expressed therefrom include using the expressed protein to adsorb circulating patient autoantibodies. These proteins, fragments of protein, or oligopeptides derived from the predicted sequence can be bound to solid phase particles over which the patient plasma may pass during plasmapheresis and extracorporeal immunoadsorption, thus reducing anti-Ro/SSA antibody levels before the remaining plasma is returned to the patient. If public idiotypes are found on anti-52 kDa Ro/SSA autoantibodies from different patients, appropriate fragments of these antibodies can be used to generate anti-idiotypic antibodies. The latter antibodies may be used either to block binding of anti-52 kDa antibodies to the native Ro/SSA particle *in vivo*, or to replace the anti-Ro/SSA

molecules discussed above for treatment involving plasmapheresis and extracorporeal immunoadsorption.

The present invention will be further described with reference to the following description of the isolation and characterization of the 52 kDa Ro/SSA protein cDNA.

Absorption of E. coli Antibodies:

Human sera from patients and controls were adsorbed against *E. coli* following lysis with the bacteriophage vector lambda gt11 to deplete naturally occurring anti *E. coli* antibodies from the sera. Briefly, five petri dishes with 30-50,000 plaque forming units (pfu) were plated with *E. coli* strain Y1090 cells on LB agar and grown for 4 hours at 42°C. Each dish was overlaid with a single nitrocellulose filter for 3 hours on one side and 2 hours on the other side at 37°C. Filters were removed and washed in TBST (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; and 0.5% Tween-20). Filters were then sequentially incubated for one hour each at room temperature with human serum diluted 1:100 in TBST containing 3% bovine serum albumin and 0.02% sodium azide. The success of these adsorptions was monitored by removing a small piece of each filter following the serum incubation, blocking with 5% nonfat dry milk in TBST, washing, and incubating with an alkaline phosphatase conjugated, goat anti-human IgG antisera (Sigma Chemical Co., St. Louis; 1:1000 dilution in TBST containing 5% nonfat dry milk). These filter pieces were then reacted with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) according to the ProtoBlot System (Promega Corp., Madison).

Screening a human cDNA library:

A human thymocyte cDNA library was purchased from Clonetech Laboratories (Palo Alto, CA.). 30,000 pfus per petri dish were plated with *E. coli* Y1090 cells on LB agar and incubated at 42°C for 4 hours. The partially lysed *E. coli* lawn was then overlaid for 3 hours at 37°C with a nitrocellulose membrane which had previously been soaked in 10 mM isopropylthio-β-galactoside (IPTG). Filters were removed, washed in TBST, incubated with an *E. coli* adsorbed patient serum containing antibodies to the 52 kDa Ro/SSA protein, and screened using the alkaline phosphatase method described above. Blue colored positive plaques were plaque purified using the same patient serum. Two clones, FI18.1 and FI19.3, were retested and found to react with sera from a panel of lupus patients which contained autoantibodies which react with the 52 kDa Ro/SSA protein as detected by Western blot assay. IPTG-induced proteins from these clones do not react with antibodies in sera from healthy controls nor patients with autoantibodies to the 60 kDa but not the 52 kDa Ro/SSA polypeptide.

Affinity Purification of Patient Antibodies:

Antibodies were affinity purified from two sources: (1) Western blotted polyacrylamide gels of human lymphocyte or HeLa cell extracts, and (2) nitrocellulose blots of IPTG-induced proteins from the plaque purified clones FI18.1 and FI19.3. For the first method, human cells were lysed by sonication in water and centrifuged to remove debris. Supernatants were reduced, heat denatured, and subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes using the method of Towbin, H. T. Staehelin, and J. Gordon Proc. Natl. Acad. Sci. USA 76:4350-4354 (1979), from which a small strip was removed,

soaked in TBST containing 5% nonfat dry milk, and allowed to react with serum from a patient containing anti-52 kDa Ro/SSA autoantibodies. The filter piece was treated with alkaline phosphatase conjugated anti-human antisera and developed with NBT and BCIP as described above to localize the section of the membrane which contained the Ro/SSA 52 kDa proteins. The corresponding section of the remainder of the membrane was excised and incubated with patient serum for 30 minutes at 37°C. Following five TBST washes, bound antibodies were eluted using a modification of a method by Krohne, G., R. Stick, J.A. Kleinschmidt, R. Moll, W.W. Frankie, and P. Hausen J.Cell. Biol. 94:749-754 (1982). The membrane was incubated with patient serum for 30 minutes at 37°C, and the bound antibodies were eluted with 3 M sodium, rather than potassium, thiocyanate. The elution process was repeated five times and the resulting solution was concentrated approximately 50-fold using a Centriprep 30 concentrator (Amicon Division, W.R. Grace, Danvers, MA). Antibodies which bound to proteins produced in IPTG-induced plaque purified bacteriophage clones FI18.1 or FI19.3, or in wild type lambda gt11 as a control, were eluted in an identical manner from nitrocellulose blots.

Affinity purified antibodies isolated from Western blots of human lymphocyte or HeLa cell extracts were tested for their ability to bind to IPTG-induced proteins in clones FI18.1 and FI19.3, and in wild type lambda gt11 transfected *E. coli* cells. Affinity purified antibodies isolated from IPTG-induced proteins of these bacteriophage clones were used to determine their binding specificity to Western blots of human lymphocyte extracts using the method of Towbin, et al. (1979). The use of affinity purified antibodies in such assays was identical to that described for patient serum above, except that the affinity purified

antibodies were used undiluted. Antibodies eluted from proteins from IPTG-induced clones FI18.1 and FI19.3, but not IPTG-induced lambda gt11 transfected *E. coli* cells, bound only to a 52 kDa protein in Western blots. Affinity purified antibody from the 52 kDa region of Western blotted lymphocyte extracts bound to IPTG-induced proteins of clones FI18.1 and FI19.3, and not IPTG-induced proteins of lambda gt11 transfected *E. coli*.

Immunoprecipitations of Ro/SSA Proteins and hY RNAs:

Affinity purified antibodies isolated from IPTG-induced proteins of clone FI18.1 or FI19.3 were used to immunoprecipitate nucleic acids from HeLa cells which are bound to Ro/SSA proteins using the technique of Forman, M.S., M. Nakamura, T. Mimori, C. Gelpi, and J.A. Hardin Arthritis Rheum. 28:1356-1361 (1985). These antibodies were bound to Staphylococcal protein-A-coated Sepharose™ CL-4B beads (Pharmacia, Piscataway, NJ). HeLa cells were lysed by sonication in the presence of 0.05% NP-40 and mixed with these beads. Following washing of the beads, bound material was eluted with 0.3 M sodium acetate and 1% SDS, and subjected to phenol/chloroform extraction. Ethanol precipitated nucleic acids were dissolved in electrophoresis sample buffer and subjected to polyacrylamide gel electrophoresis in the presence of 7 M urea, and finally were silver stained. HY RNAs were identified using affinity purified antibodies from these clones, but not from IPTG-induced lambda gt11 transfected *E. coli*.

Characterization of DNA inserts in clones FI18.1 and FI19.3:

DNA was extracted from the bacteriophage clones, digested with the restriction enzyme *Eco*RI and electrophoresed in both agarose and polyacrylamide gels. Staining with ethidium bromide revealed that

both clones contained a single 1.8 kilobase insert. DNA from each clone was subjected to electrophoresis in 0.8% agarose gels and transferred to nylon membranes (Amersham, Arlington Heights, IL) by the method of Southern, E.M. J. Mol. Biol. 98:503-517 (1978). Isolated inserts were radioactively labeled with α-^{32}P-dCTP using random hexamer primers by the procedures of Feinberg, A.P., and B. Vogelstein Anal Biochem 132:6-13 (1983) and Feinberg, A.P., and B. Vogelstein Anal. Biochem. 137:266-267 (1984), and hybridized to nylon membranes to analyze their sequence similarity in cross-hybridization studies. Following hybridization, the resulting membranes were washed under high stringency with 0.1 x SSPE (15 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM EDTA, pH 7.0) and 0.1% SDS at 65°C. Hybridization was detected by autoradiography. Cross-hybridization of inserts of clones FI18.1 and FI19.3 was detected.

The *Eco*RI inserts from each bacteriophage clone were purified following electrophoresis through preparative 5% N,N'-bis-acrylylcystamine cross-linked polyacrylamide gels (Bio-Rad Laboratories, Richmond, CA), reduction in 2-mercaptoethanol, and DEAE ion-exchange chromatography. The resulting fragments were subcloned into an *Eco*RI digested M13mp19 bacteriophage vector, as described by Yanisch-Perron, C., J. Vieira, and J. Messing Gene 33:103-119 (1985). Additional DNA from clone FI18.1 was digested with other restriction enzymes chosen for their ability to produce DNA fragments which could be directly subcloned into the multiple cloning sites of M13mp19. Following ligation of these inserts to the vectors with T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD), DNA was transformed in *E. coli* strain JM103. Single stranded DNA from M13 subclones was prepared by the method of Sanger, F.,

S. Nicklen, and A.R. Coulsen Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977), and subjected to nucleotide sequencing using the dideoxy chain-termination method of Sanger, F., A.R. Coulson, B.G. Barrell, A.J.H. Smith, and B.A. Roe J. Mol. Biol. 143:161-178 (1980) and T7 DNA polymerase, as reported by Tabor, S., and C.C. Richardson. Proc. Natl. Acad. Sci. USA 84:4767-4771 (1987) (U.S. Biochemical Corp., Cleveland, OH). Additional nucleotide sequence was determined from deletion subclones which were produced using the exonuclease activity of T4 DNA polymerase from M13 phage clones containing large inserts, using the technique of Dale, R.M.K., B.A. McClure, and J.P. Houchins Plasmid 13:31-40 (1985). The resulting nucleotide sequence of this cDNA molecule is shown in Figure 1. Computer analysis of nucleotide sequences were performed using the Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin, described by Devereux, J., P. Haeberli, and O. Smithies Nucl. Acids Res. 12:387-395 (1984), and programs written for use on the IBM PC, including modification of those described by Schwindinger, W.F., and J.R. Warner Nucl. Acids Res. 12:601-604 (1984).

Methods for expression of large quantities of protein from the cDNA. Methods for production of recombinant proteins.

In bacteria.

The protein can be expressed following IPTG induction from the lambda gt11 cDNA clone, or subclones of this cDNA in plasmids, or a portion thereof, or as modified using standard techniques, as described above.

In human or animals cell culture.

The protein can also be expressed from the cDNA, or a portion thereof, or as modified using standard techniques, following subcloning of the cDNA into a eukaryotic expression vector containing well characterized viral promoters and enhancers such as SV40 regulatory regions (Chu, G. and P.A. Sharp, Gene 13:197-202 (1981); the bovine papilloma virus (Howley, P.M., N. Sarver, and M.F. Law, Methods Enzymol. 101:357-402 (1983); or other expression vectors. These recombinant molecules can then be introduced into appropriate eukaryotic cells using the protoplast fusion technique (Schaffner, W., Proc. Natl. Acad. Sci. USA 77:2163-2167 (1986); Sadri-Goldin, R.M., et al., Methods Enzymol. 101:402-411 (1982)), $CaPO_4$ precipitation (Graham, F.L. and A.J. van der Eb Virology 52:456-457 (1973)), or other known methods. These recombinant molecules existing as either episomes, or as single or concatemeric copies integrated into chromosomal DNA can form stable transformed cells lines for expression of cDNA encoded proteins.

Following lysis of transfected bacterial or eukaryotic cells, the 52 kDa Ro/SSA protein can be purified with known methods including ion-exchange chromatography. Alternatively, the patent may be secreted from such cells depending on the host/vector system or modifications to the cDNA, and the resulting protein can be purified from cellular supernatants. The purity can be monitored by SDS-PAGE and immunodetection assays such as ELISA assays, radioimmunoassays, or Western blots.

Methods of production of antibodies to the recombinant proteins.

Polyclonal antisera produced by immunization of animals.

Animals can be immunized using standard techniques with the purified protein expressed from the cloned cDNA in order to produce polyclonal sera. Alternatively, monoclonal antibodies can be produced as follows, or using other methods also known to those skilled in the art.

Monoclonal antibodies produced by hybridomas.

BALB/c mice are injected intraperitoneally with 50-100 μg of purified protein in complete Freund's adjuvant. The mice are again immunized after 3 weeks with the protein emulsified in incomplete Freund's adjuvant and after 6 weeks with the protein in TBS (0.1 M NaCl 0.02 M Tris-HCl pH 7.5). Four days later, spleen cells are fused with the mouse myeloma cell line P3X63AG8-653 using 35% polyethylene glycol 1450, using standard techniques, as described by Laurell, M., K. Ikeda, S. Lindgren, J. Stenflo, FEBS Letters 191, 75-81 (1985); Wakabayashi, K., Y. Sakata, N. Aoki, J. Biol. Chem. 261, 11097-11105 (1986); Borrebaeck, C.A.K., M.E. Etzler, J. Biol. Chem. 256, 4723-4725 (1981); Kohler, G., C. Milstein, Nature 256, 495-497 (1975).

Cells are grown in HAT medium to select for hybridomas. After four weeks, supernatants from fused cells are screened for antibody production by solid-phase enzyme-linked immunoadsorbent assay in the presence and absence of 5 mM Ca^{2+}.

Positive clones of interest, as determined on the basis of reactivity with antigen, are recloned at least two times by limiting dilution onto murine peritoneal lavage feeder cells.

A BALB/c mouse is initially primed with pristane to induce ascites fluid production and, 14 days later, injected intraperitoneally with 0.1 ml of 10 mg/ml cyclophosphamide in order to immunocompromise the animal. Seventy-four hours later, 3-6 x 10^6 cells are injected intraperitoneally. After 7-10 days, ascites fluid is collected and monoclonal antibodies purified from ascites fluid. Antibody is normally present at 8-15 mg antibody/ml ascites fluid. Two different methods can be used to purify the antibody: (1) NH_4SO_4 fractionation followed by QAE-Sephadex chromatography; or (2) affinity chromatography on antigen bound Affi-Gel™ 10.

Alternatively, selected hybridomas can be propagated *in vitro* in laboratory culture vessels from which the monoclonal antibodies against the selected antigen can be harvested by decantation and purified as described for the ascites fluid. The antigen affinity resin can also be used to isolate the monoclonal antibodies from hybridoma tissue culture supernatants. The material is directly applied to the column. The antibody concentration in an exponentially growing culture is approximately 25 μg/ml.

Xenogeneic Antibodies Produced In Severe Combined Immunodeficient Mice.

Adult retired breeder mice C.B-17 SCID (homozygous for SCID mutation) are obtained from the Fox Chase Cancer Institute and bred and maintained in a sterile environment. At 24 weeks of age or older, mice are removed from the sterile environment and injected with human peripheral blood mononuclear cells, using the method of Mosier, D.E., R.J. Galizia, S.M. Baird, D.B Wilson Nature 335, 256-259 (1988). Mice are subsequently fed standard non-sterile mouse chow and

kept in isolation laminar flow cubicles (BioClean, Inc.). SCID mice are bled by tail vein.

Human peripheral blood mononuclear cells are obtained from patients having autoantibodies. Approximately 150 ml of blood is withdrawn into a heparinized container (preservative-free) and mononuclear cells separated by low speed density centrifugation (Histopak, Sigma Chemical Co., St. Louis, MO). Viability is determined by exclusion of trypan blue. Various numbers (x 10^6) of isolated human mononuclear cells are injected intraperitoneally.

Enzyme linked immunosorbent assays (ELISA), performed using established techniques, as described by Gaither and Harley, Protides Biol. Fluids Proc. Colloq. 33, 413 (1985), are used to screen for the production of human IgG and specific autoantibodies. To screen for human IgG production, 96 well microtiter plates are coated with mouse serum at limiting dilutions. They are subsequently washed, blocked and goat anti-human IgG (gamma chain specific) alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) added. After overnight incubation, microtiter plates are washed, substrate added and optical density readings taken on an ELISA reader (Beckman Instruments).

Engrafted mouse sera are screened for autoantibodies using a standard anti-Ro/SSA ELISA and highly purified Ro/SSA. Engrafted mouse sera is analyzed for anti-nuclear antibodies on Hep-2 cells using a NOVA Lite ANA (INOVA DIAGNOSTICS, Inc., San Diego, CA). Serum samples are diluted and 50-75 μl applied to each substrate slide. After 30 min incubation in a moist chamber at room temperature, the slides are thoroughly washed with PBS. Goat anti-human IgG gamma chain specific FITC conjugate (Sigma Chemical Co., St. Louis, MO) is

added at 1:7500 dilution and incubated for 30 min at room temperature. Slides are subsequently washed with PBS and immunofluorescent staining visualized under a fluorescence microscope.

Modifications and variations of the cDNA and protein expressed therefrom, and methods for use thereof, will be obvious to those skilled in the art from the foregoing detailed description of the invention. Such modifications and variations are intended to come within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Oklahoma Medical, Research Found

(ii) TITLE OF INVENTION: Nucleotide Sequence Encoding a 52 kDa Ro/SSA Autoantigen

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Patrea L. Pabst
(B) STREET: Kilpatrick & Cody, 100 Peachtree St., Suite 3100
(C) CITY: Atlanta
(D) STATE: Georgia
(E) COUNTRY: U.S.A.
(F) ZIP: 30303

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 07-MAY-1991
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/520,270
(B) FILING DATE: 07-MAY-1990

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: OMRF118

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 404/572-6508
(B) TELEFAX: 404/562-6555
(C) TELEX: 54-2307

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1843 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: human thymocyte
(B) CLONE: FI18.1 and FI19.3

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/520,270 u
(I) FILING DATE: 07-MAY-1990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCGGGC ACACTGCTGT TTAACGGCAC ACTTGACAAT GGCTTCAGCA GCACGCTTGA 60

CAATGATGTG GGAGGAGGTC ACATGCCCTA TCTGCCTGGA CCCCTTCGTG GAGCCTGTGA 120

GCATCGAGTG TGGCCACAGC TTCTGCCAGG AATGCATCTC TCAGGTTGGG AAAGGTGGGG 180

GCAGCGTCTG TCCTGTGTGC CGGCAGCGCT TTCTGCTCAA GAATCTCCGG CCCAATCGAC 240

AGCTAGCCAA CATGGTGAAC AACCTTAAAG AAATCAGCCA GGAGGCCAGA GAGGGCACAC 300

AGGGGGAACG GTGTGCAGTG CATGGAGAGA GACTTCACCT GTTCTGTGAG AAAGATGGGA 360

AGGCCCTTTG CTGGGTATGT GCCCAGTCTC GGAAACACCG TGACCACGCC ATGGTCCCTC 420

TTGAGGAGGC TGCACAGGAG TACCAGGAGA AGCTCCAGGT GGCATTAGGG GAACTGAGAA 480

GAAAGCAGGA GTTGGCTGAG AAGTTGGAAG TGGAAATTGC AATAAAGAGA GCAGACTGGA 540

AGAAAACAGT GGAAACACAG AAATCTAGGA TTCACGCAGA GTTTGTGCAG CAAAAAAACT 600

TCCTGGTTGA AGAAGAACAG AGGCAGCTGC AGGAGCTGGA GAAGGATGAG AGGGAGCAGC 660

TGAGAATCCT GGGGGAGAAA GAGGCCAAGC TGGCCCAGCA GAGCCAGGCC CTACAGGAGC 720

TCATCTCAGA GCTAGATCGA AGGTGCCACA GCTCAGCACT GGAACTGCTG CAGGAGGTGA 780

GAATTGTCCT GGAAAGGAGT GAGTCCTGGA ACCTGAAGGA CCTGGATATT ACCTCTCCAG 840

AACTCAGGAG TGTGTGCCAT GTGCCAGGGC TGAAGAAGAT GCTGAGGACA TGTGCAGTCC 900

ACATCACTCT GGATCCAGAC ACAGCCAATC CGTGGCTGAT ACTTTCAGAA GATCGGAGAC 960

AAGTGAGGCT TGGAGACACC CAGCACAGCA TACCTGGAAA TGAAGAGAGA TTTGATAGTT 1020

ATCCTATGGT CCTGGGTGCC CAGCACTTTC ACTCTGGAAA ACATTACTGG GAGGTAGATG 1080

TGACAGGAAA GGAGGCCTGG GACCTGGGTG TCTGCAGAGA CTCTGTGCGC AGGAAGGGGC 1140

ACTTTTTGCT TAGTTCCAAG AGTGGCTTCT GGACAATTTG GTTGTGGAAC AAACAAAAAT 1200

ATGAGGCTGG CACCTACCCC CAGACTCCCC TCCACCTTCA GGTGCCTCCA TGCCAAGTTG 1260

GGATTTTCCT GGACTATGAG GCTGGCATGG TCTCCTTCTA CAACATCACT GACCATGGCT 1320

CCCTCATCTA CTCCTTCTCT GAATGTGCCT TTACAGGACC TCTGCGGCCC TTCTTCAGTC 1380

CTGGTTTCAA TGATGGAGGA AAAAACACAG CCCCTCTAAC CCTCTGTCCA CTGAATATTG 1440

GATCACAAGG ATCCACTGAC TATTGATGGC TTTCTCTGGA CACTGCCACT CTCCCCATTG 1500

GCACCGCTTC TCAGCCACAA ACCCTGCCTC TTTTCCCCAT GAACTCTGAA CCACCTTTGT 1560

CTCTGCAGAG GCATCCGGAT CCCAGCAAGC GAGCTTTAGC AGGGAAGTCA CTTCACCATC 1620

AACATTCCTG CCCCAGATGG CTTTGTGATT CCCTCCAGTG AAGCAGCCTC CTTATATGTG 1680

GCCCAAACTC ATCTTGATCA ACCAAAAAACA TGTTTCTGCC TTCTTTATGG GACTTAAGTT 1740

TTTTTTTTCT CCTCTCCATC TCTAGGATGT CGTCTTTGGT GAGATCTCTA TTATATCTTG 1800

TATGGTTTGC AAAAGGGCTT CCTAAAAATA AAAACCCGAA TTC 1843

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 475 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: homo sapiens

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: thymocyte
(B) CLONE: FI18.1 and FI19.3

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/520,270 u
(I) FILING DATE: 07-MAY-1990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ala Ser Ala Ala Arg Leu Thr Met Met Trp Glu Glu Val Thr Cys
1               5                  10                  15

Pro Ile Cys Leu Asp Pro Phe Val Glu Pro Val Ser Ile Glu Cys Gly
            20                  25                  30

His Ser Phe Cys Gln Glu Cys Ile Ser Gln Val Gly Lys Gly Gly Gly
        35                  40                  45
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Ser Val Cys Pro Val Cys Arg Gln Arg Phe Leu Leu Lys Asn Leu Arg
    50                  55                  60

Pro Asn Arg Gln Leu Ala Asn Met Val Asn Asn Leu Lys Glu Ile Ser
65                  70                  75                  80

Gln Glu Ala Arg Glu Gly Thr Gln Gly Glu Arg Cys Ala Val His Gly
                85                  90                  95

Glu Arg Leu His Leu Phe Cys Glu Lys Asp Gly Lys Ala Leu Cys Trp
            100                 105                 110

Val Cys Ala Gln Ser Arg Lys His Arg Asp His Ala Met Val Pro Leu
        115                 120                 125

Glu Glu Ala Ala Gln Glu Tyr Gln Glu Lys Leu Gln Val Ala Leu Gly
    130                 135                 140

Glu Leu Arg Arg Lys Gln Glu Leu Ala Glu Lys Leu Glu Asx Glu Ile
145                 150                 155                 160

Ala Ile Lys Arg Ala Asp Trp Lys Lys Thr Val Glu Thr Gln Lys Ser
                165                 170                 175

Arg Ile His Ala Glu Phe Val Gln Gln Lys Asn Phe Leu Val Glu Glu
            180                 185                 190

Glu Gln Arg Gln Lys Gln Glu Leu Glu Lys Asp Glu Arg Glu Gln Leu
        195                 200                 205

Arg Ile Leu Gly Glu Lys Glu Ala Lys Leu Ala Gln Gln Ser Gln Ala
    210                 215                 220

Leu Gln Glu Leu Ile Ser Glu Leu Asp Arg Arg Cys His Ser Ser Ala
225                 230                 235                 240
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Leu Glu Leu Leu Gln Glu Val Ile Ile Val Leu Glu Arg Ser Glu Ser
                245                 250                 255

Trp Asn Leu Lys Asp Leu Asp Ile Thr Ser Pro Glu Leu Arg Ser Val
            260                 265                 270

Cys His Val Pro Gly Leu Lys Lys Met Leu Arg Thr Cys Ala Val His
        275                 280                 285

Ile Thr Leu Asp Pro Asp Thr Ala Asn Pro Trp Leu Ile Leu Ser Glu
    290                 295                 300

Asp Arg Arg Gln Val Arg Leu Gly Asp Thr Gln Gln Ser Ile Pro Gly
305                 310                 315                 320

Asn Glu Glu Arg Phe Asp Ser Tyr Pro Met Val Leu Gly Ala Gln His
                325                 330                 335

Phe His Ser Gly Lys His Tyr Trp Glu Val Asp Val Thr Gly Lys Glu
            340                 345                 350

Ala Trp Asp Leu Gly Val Cys Arg Asp Ser Val Arg Arg Lys Gly His
        355                 360                 365

Phe Leu Leu Ser Ser Lys Ser Gly Phe Trp Thr Ile Trp Leu Trp Asn
    370                 375                 380

Lys Gln Lys Tyr Glu Ala Gly Thr Tyr Pro Gln Thr Pro Leu His Leu
385                 390                 395                 400

Gln Val Pro Pro Cys Gln Val Gly Ile Phe Leu Asp Tyr Glu Ala Gly
                405                 410                 415

Met Val Ser Phe Tyr Asn Ile Thr Asp His Gly Ser Leu Ile Tyr Ser
            420                 425                 430
```

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Phe Ser Glu Cys Ala Phe Thr Gly Pro Leu Arg Pro Phe Phe Ser Pro
        435                 440                 445

Gly Phe Asn Asp Gly Gly Lys Asn Thr Ala Pro Leu Thr Leu Cys Pro
    450                 455                 460

Leu Asn Ile Gly Ser Gln Gly Ser Thr Asp Tyr
465                 470                 475
```

We claim:

1. Isolated nucleic acid sequence encoding a Ro/SSA autoantigen having a molecular weight by SDS-PAGE and Western blot of 52 kDa.

2. The sequence of claim 1 further comprising regulatory sequences selected from the non-protein coding sequences shown in Figure 1.

3. The sequence of claim 1 consisting essentially of the protein encoding sequence shown in Figure 1, after the first 38 bases.

4. The sequence of claim 2 comprising the entire sequence shown in Figure 1.

5. The sequence of claim 1 isolated from the human chromosome.

6. The sequence of claim 5 further comprising regulatory sequences for expression of the 52 kDa Ro/SSA autoantibody.

7. The sequence of claim 1 labelled with a detectable label.

8. The sequence of claim 1 hybridizing under standard conditions to the sequence shown in Figure 1, or portions thereof.

9. An isolated Ro/SSA autoantigen having a molecular weight by SDS-PAGE and Western blot of 52 kDa, or an antigenic portion thereof.

10. The protein of claim 9 having the sequence shown in Figure 2, or a portion thereof.

11. The protein of claim 9 produced by proteolytic cleavage of the protein expressed from the nucleotide sequence shown in Figure 1 or a sequence hybridizing under standard conditions to the nucleic acid shown in Figure 1.

12. The protein of ciaim 9 produced synthetically based on the nucleotide sequence shown in Figure 1 or a sequence hybridizing under standard conditions to the nucleic acid shown in Figure 1.

13. The protein of claim 9 expressed by cells selected from the group consisting of bacteria, yeast, and animal cells.

14. The protein of claim 9 expressed by an *in vitro* cell free translation system.

15. The protein of claim 9 further comprising a detectable label.

16. The protein of claim 9 immobilized on a substrate not specifically binding antibodies.

17. A method for determining the presence of antibodies reactive with a Ro/SSA protein having a molecular weight by SDS-PAGE of 52 kDa comprising providing a reactive portion of an isolated 52 kDa Ro/SSA protein encoded by the protein encoding sequence shown in Figure 1 or a nucleic sequence hybridizing thereto under standard conditions.

18. The method of claim 17 wherein the protein is isolated by binding to antibodies reactive with a 52 kDa protein encoded by the sequence shown in Figure 1, or a sequence hybridizing thereto under standard conditions, and not reactive with other Ro/SSA proteins.

19. The method of claim 17 wherein the protein is expressed from an isolated nucleic sequence encoding a 52 kDa Ro/SSA autoantigen as shown in Figure 1, or a sequence hybridizing thereto under standard conditions, and said protein is chemically different from the naturally produced and isolated autoantigen.

20. The method of claim 17 wherein the protein is expressed in an *in vitro* cell free translation system.

21. The method of claim 17 wherein the protein is expressed in cells selected from the group consisting of bacteria, yeast, and animal cells.

22. The method of claim 17 wherein the reactive portion is a proteolytically cleaved fragment of the protein encoded by the sequence of Figure 1, or a nucleic sequence hybridizing thereto.

23. The method of claim 17 wherein the reactive portion is a chemically synthesized fragment of the protein encoded by the sequence of Figure 1, or a nucleic sequence hybridizing thereto.

24. The method of claim 17 wherein the presence of the anti-52 kDa Ro/SSA autoantibodies is determined by detecting a label bound to the protein.

25. The method of claim 17 wherein the presence of the anti-52 kDa Ro/SSA autoantibodies is determined by reacting a solution to be tested with the 52 kDa protein to bind anti-52 kDa protein antibodies to 52 kDa protein, removing the unbound materials, and detecting the presence of antibody bound protein.

26. The method of claim 25 wherein the antibody is in solution and the 52 kDa protein is immobilized on a substrate not specifically binding antibody.

27. The method of claim 25 wherein the antibody bound protein is separated by binding with a reagent specifically binding to antibody.

28. The method of claim 25 further comprising quantitating the amount of antibody bound protein.

29. A method for treating a patient comprising exposing the blood of a patient characterized by Ro/SSA autoantibodies to a 52 kDa protein to a protein selected from the group consisting of 52 kDa

Ro/SSA protein, 52 kDa Ro/SSA fragments, 52 kDa Ro/SSA oligopeptides, and anti-idiotypic antibodies to 52 kDa Ro/SSA antibodies.

30. The method of claim 29 wherein the proteins are immobilized on a substrate in an apparatus for extracorporeal treatment of blood.

31. A protein binding with an antibody to a protein expressed by the nucleotide sequence shown in Figure 1 and hybridizing to the nucleotide sequence shown in Figure under standard conditions.

```
          10         20         30         40         50         60
GAATTCGGGC ACACTGCTGT TTAACGGCAC ACTTGACAAT GGCTTCAGCA GCACGCTTGA

          70         80         90        100        110        120
CAATGATGTG GGAGGAGGTC ACATGCCCTA TCTGCCTGGA CCCCTTCGTG GAGCCTGTGA

         130        140        150        160        170        180
GCATCGAGTG TGGCCACAGC TTCTGCCAGG AATGCATCTC TCAGGTTGGG AAAGGTGGGG

         190        200        210        220        230        240
GCAGCGTCTG TCCTGTGTGC CGGCAGCGCT TTCTGCTCAA GAATCTCCGG CCCAATCGAC

         250        260        270        280        290        300
AGCTAGCCAA CATGGTGAAC AACCTTAAAG AAATCAGCCA GGAGGCCAGA GAGGGCACAC

         310        320        330        340        350        360
AGGGGGAACG GTGTGCAGTG CATGGAGAGA GACTTCACCT GTTCTGTGAG AAAGATGGGA

         370        380        390        400        410        420
AGGCCCTTTG CTGGGTATGT GCCCAGTCTC GGAAACACCG TGACCACGCC ATGGTCCCTC

         430        440        450        460        470        480
TTGAGGAGGC TGCACAGGAG TACCAGGAGA AGCTCCAGGT GGCATTAGGG GAACTGAGAA

         490        500        510        520        530        540
GAAAGCAGGA GTTGGCTGAG AAGTTGGAAG TGGAAATTGC AATAAAGAGA GCAGACTGGA

         550        560        570        580        590        600
AGAAAACAGT GGAAACACAG AAATCTAGGA TTCACGCAGA GTTTGTGCAG CAAAAAAACT

         610        620        630        640        650        660
TCCTGGTTGA AGAAGAACAG AGGCAGCTGC AGGAGCTGGA GAAGGATGAG AGGGAGCAGC

         670        680        690        700        710        720
TGAGAATCCT GGGGGAGAAA GAGGCCAAGC TGGCCCAGCA GAGCCAGGCC CTACAGGAGC

         730        740        750        760        770        780
TCATCTCAGA GCTAGATCGA AGGTGCCACA GCTCAGCACT GGAACTGCTG CAGGAGGTGA

         790        800        810        820        830        840
TAATTGTCCT GGAAAGGAGT GAGTCCTGGA ACCTGAAGGA CCTGGATATT ACCTCTCCAG

         850        860        870        880        890        900
AACTCAGGAG TGTGTGCCAT GTGCCAGGGC TGAAGAAGAT GCTGAGGACA TGTGCAGTCC

         910        920        930        940        950        960
ACATCACTCT GGATCCAGAC ACAGCCAATC CGTGGCTGAT ACTTTCAGAA GATCGGAGAC

         970        980        990      1,000      1,010      1,020
AAGTGAGGCT TGGAGACACC CAGCAGAGCA TACCTGGAAA TGAAGAGAGA TTTGATAGTT
```

FIGURE 1

```
      1,030       1,040       1,050       1,060       1,070       1,080
ATCCTATGGT CCTGGGTGCC CAGCACTTTC ACTCTGGAAA ACATTACTGG GAGGTAGATG

      1,090       1,100       1,110       1,120       1,130       1,140
TGACAGGAAA GGAGGCCTGG GACCTGGGTG TCTGCAGAGA CTCTGTGCGC AGGAAGGGGC

      1,150       1,160       1,170       1,180       1,190       1,200
ACTTTTTGCT TAGTTCCAAG AGTGGCTTCT GGACAATTTG GTTGTGGAAC AAACAAAAAT

      1,210       1,220       1,230       1,240       1,250       1,260
ATGAGGCTGG CACCTACCCC CAGACTCCCC TCCACCTTCA GGTGCCTCCA TGCCAAGTTG

      1,270       1,280       1,290       1,300       1,310       1,320
GGATTTTCCT GGACTATGAG GCTGGCATGG TCTCCTTCTA CAACATCACT GACCATGGCT

      1,330       1,340       1,350       1,360       1,370       1,380
CCCTCATCTA CTCCTTCTCT GAATGTGCCT TTACAGGACC TCTGCGGCCC TTCTTCAGTC

      1,390       1,400       1,410       1,420       1,430       1,440
CTGGTTTCAA TGATGGAGGA AAAAACACAG CCCCTCTAAC CCTCTGTCCA CTGAATATTG

      1,450       1,460       1,470       1,480       1,490       1,500
GATCACAAGG ATCCACTGAC TATTGATGGC TTTCTCTGGA CACTGCCACT CTCCCCATTG

      1,510       1,520       1,530       1,540       1,550       1,560
GCACCGCTTC TCAGCCACAA ACCCTGCCTC TTTTCCCCAT GAACTCTGAA CCACCTTTGT

      1,570       1,580       1,590       1,600       1,610       1,620
CTCTGCAGAG GCATCCGGAT CCCAGCAAGC GAGCTTTAGC AGGGAAGTCA CTTCACCATC

      1,630       1,640       1,650       1,660       1,670       1,680
AACATTCCTG CCCCAGATGG CTTTGTGATT CCCTCCAGTG AAGCAGCCTC CTTATATTTG

      1,690       1,700       1,710       1,720       1,730       1,740
GCCCAAACTC ATCTTGATCA ACCAAAAAAC TGTTTCTGCC TTCTTTATGG GACTTAAGTT

      1,750       1,760       1,770       1,780       1,790       1,800
TTTTTTTTCT CCTCTCCATC TCTAGGATGT CGTCTTTGGT GAGATCTCTA TTATATCTTG

      1,810       1,820       1,830       1,840       1,850
TATGGTTTGC AAAAGGGCTT CCTAAAAATA AAAACCCGAA TTC
```

FIGURE 1 CONTINUED

```
          5         10        15        20        25        30
M A S A A R L T M M W E E V T C P I C L D P F V E P V S I E

         35        40        45        50        55        60
C G H S F C Q E C I S Q V G K G G G S V C P V C R Q R F L L

         65        70        75        80        85        90
K N L R P N R Q L A N M V N N L K E I S Q E A R E G T Q G E

         95       100       105       110       115       120
R C A V H G E R L H L F C E K D G K A L C W V C A Q S R K H

        125       130       135       140       145       150
R D H A M V P L E E A A Q E Y Q E K L Q V A L G E L R R K Q

        155       160       165       170       175       180
E L A E K L E V E I A I K R A D W K K T V E T Q K S R I H A

        185       190       195       200       205       210
E F V Q Q K N F L V E E E Q R Q L Q E L E K D E R E Q L R I

        215       220       225       230       235       240
L G E K E A K L A Q Q S Q A L Q E L I S E L D R R C H S S A

        245       250       255       260       265       270
L E L L Q E V I I V L E R S E S W N L K D L D I T S P E L R

        275       280       285       290       295       300
S V C H V P G L K K M L R T C A V H I T L D P D T A N P W L

        305       310       315       320       325       330
I L S E D R R Q V R L G D T Q Q S I P G N E E R F D S Y P M

        335       340       345       350       355       360
V L G A Q H F H S G K H Y W E V D V T G K E A W D L G V C R

        365       370       375       380       385       390
D S V R R K G H F L L S S K S G F W T I W L W N K Q K Y E A

        395       400       405       410       415       420
G T Y P Q T P L H L Q V P P C Q V G I F L D Y E A G M V S F

        425       430       435       440       445       450
Y N I T D H G S L I Y S F S E C A F T G P L R P F F S P G F

        455       460       465       470       475
N D G G K N T A P L T L C P L N I G S Q G S T D Y
```

FIGURE 2

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03139

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07 H 15/12
U.S. Cl.: 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

Automated patent search system USPAT, commercial computer data banks SIN and DIALOG

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF CLINICAL INVESTIGATIONS, VOLUME 83, NUMBER 4, ISSUED 1989, E. BEN-CHETRIT ET AL., "ISOLATION AND CHARACTERIZATION OF A COMPLEMENTARY DNA CLONE ENCODING THE 60-KD COMPONENT OF THE SS-A-RO RIBONUCLEOPROTEIN AUTOANTIGEN", PAGES 1284-1292; ABSTRACT ONLY.	1-28
A	WO, A, 89/09273 (UNIVERSITY OF TEXAS-SYS.) 10 MAY 1989; ABSTRACT ONLY.	1-28

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 July 1991	19 AUG 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Bradley L. Sisson

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-8 telephone practice

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Detailed Reasons for Holding Lack of Unity of Invention:

I. Claims 1-8, drawn to nucleic acids, classified in Class 536, subclass 27.

II. Claims 9-28 and 31, drawn to proteins and their use in immunoassays, classified in Class 435, subclass 7.1.

III. Claims 29-30, drawn to extracorporeal treatment, classified in Class 604, subclass 5.

Detailed Reasons for Holding Lack of Unity of Invention (Continued):

The claims of these three groups are drawn to distinct inventions which are not linked so as to form a single inventive concept. PCT Rule 13.1 and 13.2 do not provide for multiple products and methods.